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(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; University Offices, Wellington Square, Oxford OX1 2JD (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SHCHEPINOV, Mikhail Sergeevich [RU/GB]; University of Oxford, Department of Biochemistry, South Parks Road, Oxford OX1 3QU (GB). SOUTHERN, Edwin, Mellor [GB/GB]; University of Oxford, Department of Biochemistry, South Parks Road, Oxford OX1 3QU (GB).

(74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).

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(57) Abstract

Interactions between a target polynucleotide and an immobilised complementary polynucleotide can be made stable at high temperatures by the use of multimeric structures. Typically, the multimeric structures are dendrimers that terminate with the same polynucleotide sequence.

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POLYNUCLEOTIDE MULTIMERS AND THEIR USE IN HYBRIDISATION ASSAYS

Field of the Invention

This invention relates to polynucleotide complexes and their use in hybridisation assays.

Background of the Invention

In assays designed to elicit qualitative (yes/no) or quantitative information about polynucleotides, a hybridisation reaction with a complementary sequence is often a key procedure. For example, hybridisation reactions may provide sequence information on a DNA molecule, or may be useful for the detection of point mutations. In order to conduct such assays on an ever-increasing commercial scale, "DNA arrays" have been produced, comprising many different polynucleotides immobilised in a dense array. The amount of nucleic acid information encoded on the array, in the form of different probes, is limited only by the physical size of the array and, say, lithographic resolution.

Typically, the immobilised polynucleotides are relatively short sequences of single-stranded nucleic acids. The nucleic acid sequences that are used to hybridise to these are however comparatively long. The stability of a duplex formed between the immobilised polynucleotides and the target nucleic acids is characterised by so-called "melting temperature" (Tm), which is the mid-point of the temperature range at which the duplex associates. Up to a point, this temperature increases with the increase in the length of the duplex. However, one problem that arises during the hybridisation reaction is the formation of intramolecular base pairs that occur between complementary sequences in the long (target) strand. If the intramolecular duplex is more stable than that formed between the immobilised polynucleotide and target nucleic acids, then it becomes impossible to analyse these regions.

Dendrimers are a known class of compounds. In general terms, they are obtainable by the reaction of a core molecule having at least two reactive groups sequentially with doubling, trebling or more, branching synthons and optionally also with non-branching synthons, to provide a multimeric structure having branched units and optionally also non-branched units. Methods for their production are described in International Patent Application No. PCT/GB98/02578.

Summary of the Invention

The present invention is based on the discovery that interactions between a target polynucleotide and an immobilised complementary polynucleotide can be made stable at high temperatures by the use of multimeric structures.

There is therefore a dendrimer structure having branches that terminate with the same polynucleotide sequence. The polynucleotide is then able to hybridise to complementary polynucleotides on an array, or on additional dendrimers.

The polynucleotide sequence on the dendrimer may be any sequence of interest. In an embodiment of the invention, the terminal polynucleotides of the same sequence are poly-A, poly-T, poly-G or poly-C.

In a preferred embodiment of the invention, multiple target polynucleotides are each attached to a branch of a dendrimer. This polynucleotide dendrimer is then able to undergo multiple interactions on a polynucleotide array. The hybridisation reaction may then be carried out at elevated temperatures resulting in the disruption of intramolecular interactions which permits further interaction with the array.

In an embodiment of the invention, the dendrimer may be immobilised on a solid support. Immobilisation may occur through a hybridisation reaction with a complementary, immobilised polynucleotide molecule.

The present invention also relates to the use of a multimeric polynucleotide for hybridisation interaction.

In this use, the multimeric polynucleotide may be a dendrimer as defined above.

There is also an assay for a target polynucleotide by hybridisation with an immobilised polynucleotide, comprising the preliminary step of conjugating the target to a dendrimer having a plurality of terminal groups reactive therewith. The immobilised polynucleotide may be of known sequence. The hybridisation assay may then be carried out at a temperature sufficient to disrupt intramolecular interactions.

In a related embodiment, the dendrimer used in the assay comprises an additional polynucleotide which is capable of hybridising to a complementary polynucleotide on one further dendrimer.

In a separate embodiment, there may be more than one target polynucleotide conjugated to the dendrimer.

Description of the Drawings

The invention will be described by way of example only with reference to the accompanying drawings, in which:

Figure 1(a) and (b) illustrates the co-operative interactions that occur when multimeric polynucleotides are hybridised to a polynucleotide array;

Figure 2 illustrates favourable and unfavourable orientations of polynucleotide dendrimers with respect to a polynucleotide array; and

Figure 3 illustrates the various supradendritic structures that may be formed between different dendrimers through secondary interactions, to bring together multiple target nucleic acids.

Description of the Invention

The present invention is based on the realisation that the stability of a duplex between complementary polynucleotides can be increased when multimeric polynucleotides are used. The term "multimeric" is used in the context of the invention to define an ability between two components to undergo interactions between multiple components. In particular, the term refers to multiple

polynucleotides interconnected either through their binding on a solid support, to a dendrimer structure, or in a tandem linkage.

The term "melt" is used to describe dissociation of a polynucleotide from its complement at elevated temperatures.

The term "polynucleotide" is used to denote a sequence of nucleic acids. The term is applied typically to DNA but also encompasses synthetic derivatives that undergo hybridisation reactions e.g. PNA.

The use of multimeric polynucleotides allows multiple hybridisation reactions to occur with a resulting increase in the stability of the hybridised components compared to a duplex formed between polynucleotide monomers. This increase in stability is characterised by higher melting temperatures and higher temperatures of reassociation exhibited by the multimeric polynucleotides in comparative tests with polynucleotide monomers.

Without wishing to be bound by theory, it would seem that the increased stability exhibited by multimeric polynucleotides may be due to the ability of the multimer to maintain any dissociated polynucleotides in close proximity to the components in the array where duplex formation may re-occur. In contrast, polynucleotide monomers which dissociate from the array are not maintained in proximity to the array, and so duplex formation can only re-occur if the dissociated polynucleotide has diffused back to the surface of the array. This is a slow process. The stability of the duplexes formed between multimeric polynucleotides may therefore correlate with the potential number of duplexes which can occur. The principle of a cooperative mode of interaction is shown in Figure 1 which shows how a multimeric structure may hold dissociated polynucleotides in close proximity to an array.

Dendrimers are a preferred form of multimeric polynucleotide, but multimerisation can be achieved in other ways which will also provide multiple interactions

with polynucleotides bound to a solid support. For example, double-stranded DNA molecules can be joined together by DNA ligase to form concatemers. PCR products could be joined in this way. In this case, the primers used for the PCR would, preferably, include the sequence of a type II restriction enzyme which produced sticky ends on cleavage, facilitating ligation of the fragments.

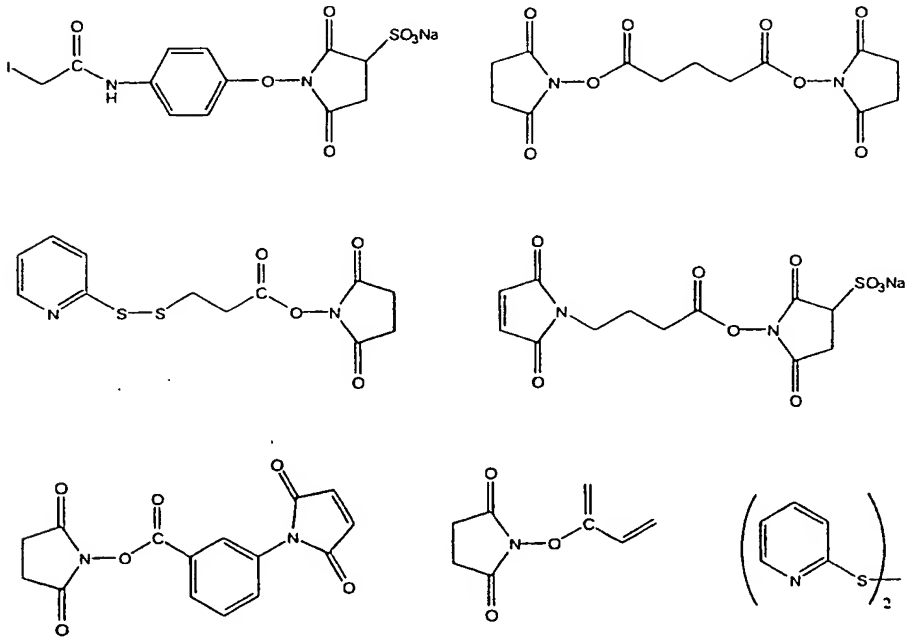
To increase the formation of stable duplexes of dendritic polynucleotides, it is important to consider the orientation of the polynucleotide on the dendrimer. It is also important that duplex formation occurs with spatial separation between the dendrimer structure and the array. For example, Figure 2 shows how orientation may affect the formation of the duplex. In Figure 2(a), hybridisation between polynucleotides bound to an array at the 5'-end, and target polynucleotides connected at the 3'-end to a dendrimer, will orientate the dendrimer in close proximity to the solid support of the array. This has a negative effect on the number of hybridisation reactions that potentially can occur. In contrast, in (b), if both the target polynucleotide and the polynucleotide on the array are attached at the same end (5' or 3'), the dendritic core is spatially orientated away from the solid support of the array, resulting in more favourable duplex formation.

In an embodiment of the invention, single copies of polynucleotides can be hybridised to an array at high temperatures, provided they can be assembled into supramolecular structures. The duplex between a target and immobilised polynucleotide may be stabilised through multiple interactions occurring on different dendrimers. For example, Figure 3 shows how complementary polynucleotides on different dendrimers may hybridise together to bring multiple target polynucleotides into a multimeric structure. This allows the targets to make multiple contact with the array. Separate dendrimers may be stabilised through interactions occurring with complementary polynucleotide dendrimers (a), (b) and (c),

or through interactions with a single-stranded polynucleotide (d). These auxiliary interactions may be carried out, for example, using simple polyA, polyT, polyG or polyC branches.

Dendrimers used in the present invention are available commercially or alternatively may be produced by the methods described in International Patent Application No. PCT/GB98/02578. In one embodiment of the invention, the dendrimer may contain a colloidal nanoparticle, eg gold, as the dendritic core.

The polynucleotide may be incorporated onto the dendrimer either by chemical means using commercially available cross-linking and activating reagents. Suitable commercially available cross-linking and activating reagents include:



Alternatively, an enzymatic process may be used whereby polynucleotide primers attached to a dendrimer are used in a polymerase chain reaction to generate the desired single-stranded polynucleotide. Alternatively, a DNA ligase may be used to link a single stranded polynucleotide to a short sequence on the dendritic core. The dendritic core may be synthesised on a solid support and conjugation of the single stranded polynucleotides may take place while the dendrimer is attached to the support, or may take place in solution. Commercially available polyaminated dendrimers may also be used.

The following Examples illustrate the invention.

Example 1

A series of 140-150 base polynucleotide strands were synthesised on a 250nm derivatised CPG support (Glen) as described previously in International Patent Application No. PCT/GB98/02578 using 2 condensations with trebling reagent so that each dendrimer comprised nine arms. Each dendrimer arm carried a 15-mer polynucleotide having the sequence:

5'-TTCTTTCTCTTCCCT SEQ ID NO.1

Each polynucleotide was linked to the dendrimer core using 3'-phosphoramidites. The sequence was labelled using 3.7 x $10^5 S^{-1}$ (10μ Ci) of γ-^{32}P-ATP [(10μ Ci/μl, 3000 pmol/ml)] and 10 units of T_4 polypolynucleotide kinase at 37°C for 30 minutes. A single-stranded polynucleotide was labelled similarly and used as the control. This polynucleotide had the sequence:

5'-TTTCTCTTTCTCTTC SEQ ID NO.2

The labelled products were purified by spinning through a TE-10 polynucleotide purification column.

Polynucleotide arrays complementary to either the control polynucleotide or the polynucleotide dendrimer were synthesized using the procedure described in Shchepinov *et al*, Nucl.Acids Res.(1997) 25: 1155-1161, using aminated polypropylene as a solid support. Both 3'- and 5'-phosphoramidites were used to link the polynucleotides to

the array, resulting in different orientations for the immobilised polynucleotides.

Hybridisation reactions were carried out overnight at 30°C in TMA buffer (3.5 M TMA, 50 mM Tris-Cl pH 8, 0.2 mM EDTA, 0.04 mg/ml SDS) using equimolar amounts of the dendrimer and control. After washing under the same conditions, the arrays were exposed to a phosphor screen (Fuji STIII) which was then scanned using a phosphorimager.

When compared to the control, the results showed a proportional (x8) increase in the signal intensity on the array for the duplex formed between the dendritic polynucleotide and the polynucleotide connected to the support through its 3'-ends. The array containing polynucleotides connected through the 5'-end showed a two-fold increase in signal intensity compared to that of the control, suggesting an unfavourable orientation of the dendrimer.

Example 2

In a further experiment, the arrays and hybridised polynucleotides made according to Example 1, were treated at elevated temperatures. Temperature increases of 2.5°C were used to establish the average temperature (Tm) at which the polynucleotide dendrimer or control melted off from the array. The Tm was found to be 57.5°C for the oligodendrimers and 45°C for the control.

Additional experiments were carried out using higher generation dendrimers. These dendrimers comprised 27 branches, synthesized by three condensations with a trebling reagent and subsequent synthesis of a 15-mer polynucleotide as in Example 1, conjugated to each branch. The melting temperature was found to be similar to the nine branched dendrimers, however the shape of the melting curve was less steep, so that at temperatures well above Tm, the amount of the dendrimer maintained on the array was considerably higher.

When the temperature was increased beyond Tm, it was found that hybridisation could still be detected for the

nine-branched dendrimer at 70°C, and 72.5°C for the 27-branched dendrimer. In contrast, no hybridisation could be detected for the control.

Example 3

To test the stability of hybridisation of a single stranded polynucleotide dendrimer to an array, a first and second set of 20-mer dendrimers were made each having the target sequence:

5'-TCTCTTTCCCTTCCTTCCTC SEQ ID NO.3

Each dendrimer contained a dendritic cap at its 5' end comprising nine 15-mer oligothymidylates. Both sets of dendrimers were hybridised to an array synthesised according to Southern *et al.*, Nucl. Acids Res. (1994) 22: 1368-1373, and comprising different lengths (1-20) of complementary sequences.

The first set of dendrimers was stabilised on the array using an auxiliary dendrimer (27-branched dendrimer) having a 15-mer oligoadenylate attached to each branch. The second set was stabilised using polyA (Sigma). In both cases, for hybridisation at 40°C, the shortest length of the polynucleotide on the array that could support hybridisation was 11, whereas for the control, it was 17-18.

Example 4

To test whether the dendrimer structures could be used to avoid intramolecular base pairing during hybridisation, second and third generation dendrimers (9 or 27 branches) were synthesized having a 137-mer polynucleotide attached to each branch at the 3'-end. The polynucleotide has the sequence:

5'-TACAGCAAATGCTTGCTAGACCAATAATTAGTTATTCACCTTGCTAAAGAAATT
CTTGCTCGTTGACCTCCACTCAGTGTGATTCCACCTTCTCCAAGAACTATATTGTCT
TTCTCTGCAAACTTGGAGATGTCCTA SEQ ID NO.4

This sequence was also used as the single-stranded monomeric control. A polynucleotide array was synthesized according to Southern *et al.*, (*supra*), having the sequence:

5'-AGTGGAGGTCAACGAGCAAGAATTTCTTTAGCAAGG SEQ ID NO.5

For the control, no hybridisation occurs at room temperature and 40°C. However, hybridisation can be detected for the dendrimers at 40°C, and at 45°C hybridisation with the array occurs in almost all 21-mers. Hybridisation is also detected at elevated temperatures, e.g. 65°C.

Example 5

A series of compounds bearing long polynucleotide branches were made using chemically presynthesised dendritic core structures and chemically synthesised single-stranded polynucleotides or PCR products, bearing terminal reactive amino- or mercaptogroups.

To achieve this, the dendritic core was assembled as described in Example 1. Blocking groups - dimethoxytrityl (DMTr) - present on the functional groups were removed and terminal OH-groups were condensed with aminolink phosphoramidite or disulphide-containing phosphoramidite to give corresponding amino- or mercaptoderivatives, after appropriate deprotection. The synthesis was carried out using increased condensation time (5-7 min). 0.02M iodine solution was used instead of 0.1M in the case of disulphide-containing phosphoramidite. Polynucleotides were labelled with reporter groups as described in Example 1. Condensation of the 5'-end with 'phosphate-ON'-phosphoramidite in combination with Beaucage reagent was used to synthesise polynucleotides bearing 5'-thiophosphate groups (for subsequent alkylation by alkylhalides-for example, Sulfo-SIAB (Pierce).

Example 6

137-mer bearing 3'-mercaptogroup connected to it through pentaethyleneglycol linker was synthesised on 250nm T-derivatised LCAA-CPG starting from C_6-disulphide reagent (Glen). The pentaethyleneglycol linker was then attached and followed by standard synthesis of 137-mer (0.02 M iodine oxidation), which was purified on HPLC by DMTr after

ammonolysis in presence of DTT, and finally detritylated. All procedures involving SH-derivatised polynucleotides were conducted according to Glen recommendations. Polynucleotides with SH-groups were then treated with either sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (Sulfo-SMPB) or N-(γ-maleimidobutyryloxy)sulfosuccinimide ester (GMBS) according to manufacturer (Pierce) to activate the SH-group. These compounds were then reacted with amino-derivatised dendritic structures in solution (ammonolysis after detritylation, evaporated and used without purification) according to Pierce protocols using 5-10 eqv. of activated polynucleotide per branch (i.e., about 250 eqv. for the third generation dendrimer). Other commercially available cross-linking reagents (Pierce) were also used to give dendritic structures with yields generally higher than in Example 1.

Example 7

Colloidal gold particles of different sizes (10 and 15nm; Nanoprobes) were also used as dendritic cores, utilising mercapto-derivatized polynucleotides or PCR products. Immobilisation conditions were essentially as described by Mirkin *et al*; Nature (1996) 382; 607-609. Excess of polynucleotide was used (x 300-2000) compared to colloidal gold particles. The compounds obtained did not move through PAGE but had some mobility on 1% agarose gel.

CLAIMS

1. A dendrimer having branches that terminate with the same polynucleotide sequence.

2. A dendrimer according to claim 1, comprising at least 3 copies of said sequence.

3. A dendrimer according to claim 1 or claim 2, wherein the dendrimer is a colloidal particle.

4. A dendrimer according to any preceding claim, wherein said same polynucleotide sequence is poly-A, poly-T, poly-G or poly-C.

5. A dendrimer according to any preceding claim, which is immobilised.

6. A dendrimer according to any of claims 1 to 4, comprising also at least one branch that terminates with a different polynucleotide sequence.

7. A dendrimer according to claim 6, which is bound to a solid surface by hybridisation of said different sequence to a complementary, immobilised polynucleotide molecule.

8. Use of a multimeric polynucleotide for hybridisation interaction.

9. Use according to claim 8, wherein the multimeric polynucleotide is a dendrimer according to any of claims 1 to 7.

10. An assay for a target polynucleotide by hybridisation with an immobilised polynucleotide, which comprises the preliminary step of conjugating said target to a dendrimer having a plurality of terminal groups reactive therewith.

11. An assay according to claim 10, wherein hybridisation occurs at a temperature sufficient to disrupt intramolecular hybridisation.

12. An assay according to claim 10 or claim 11, wherein the dendrimer comprises an additional polynucleotide which hybridises to a complementary polynucleotide on one further dendrimer.

13. An assay according to claim 10 or claim 11, wherein the hybridisation additionally occurs between a further polynucleotide conjugated to the dendrimer, and a single-stranded polynucleotide monomer.

14. An assay according to claim 10 or claim 11, wherein more than one target polynucleotide is conjugated to the dendrimer.

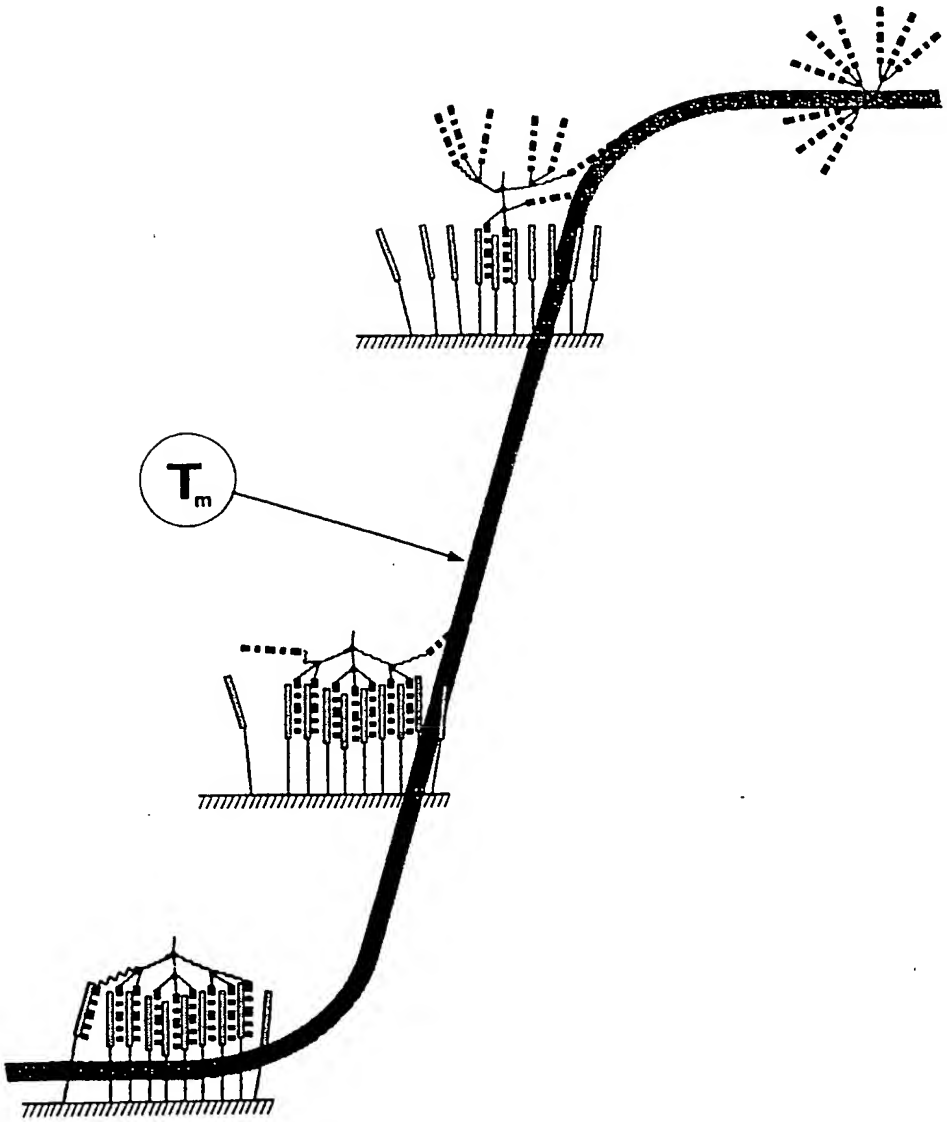


Fig. 1(a)

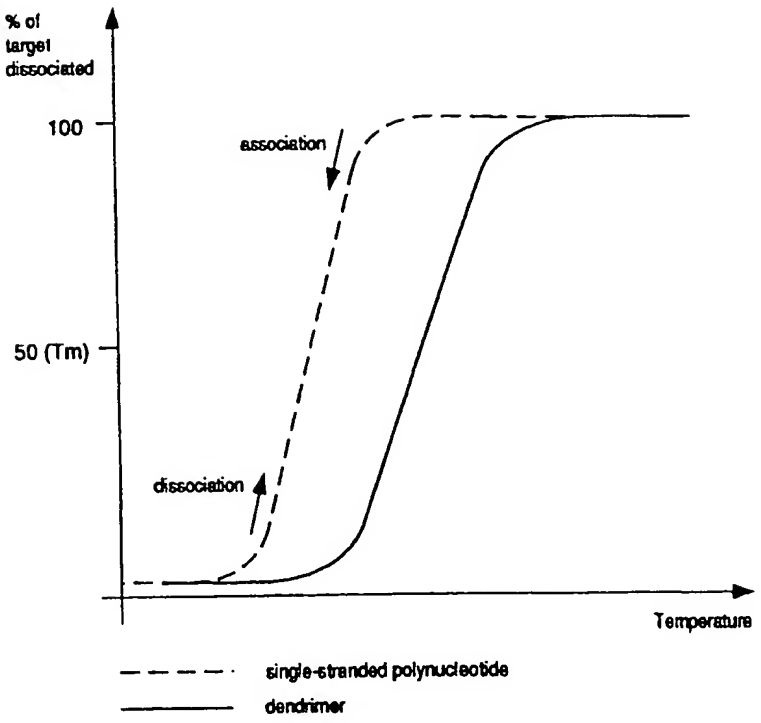
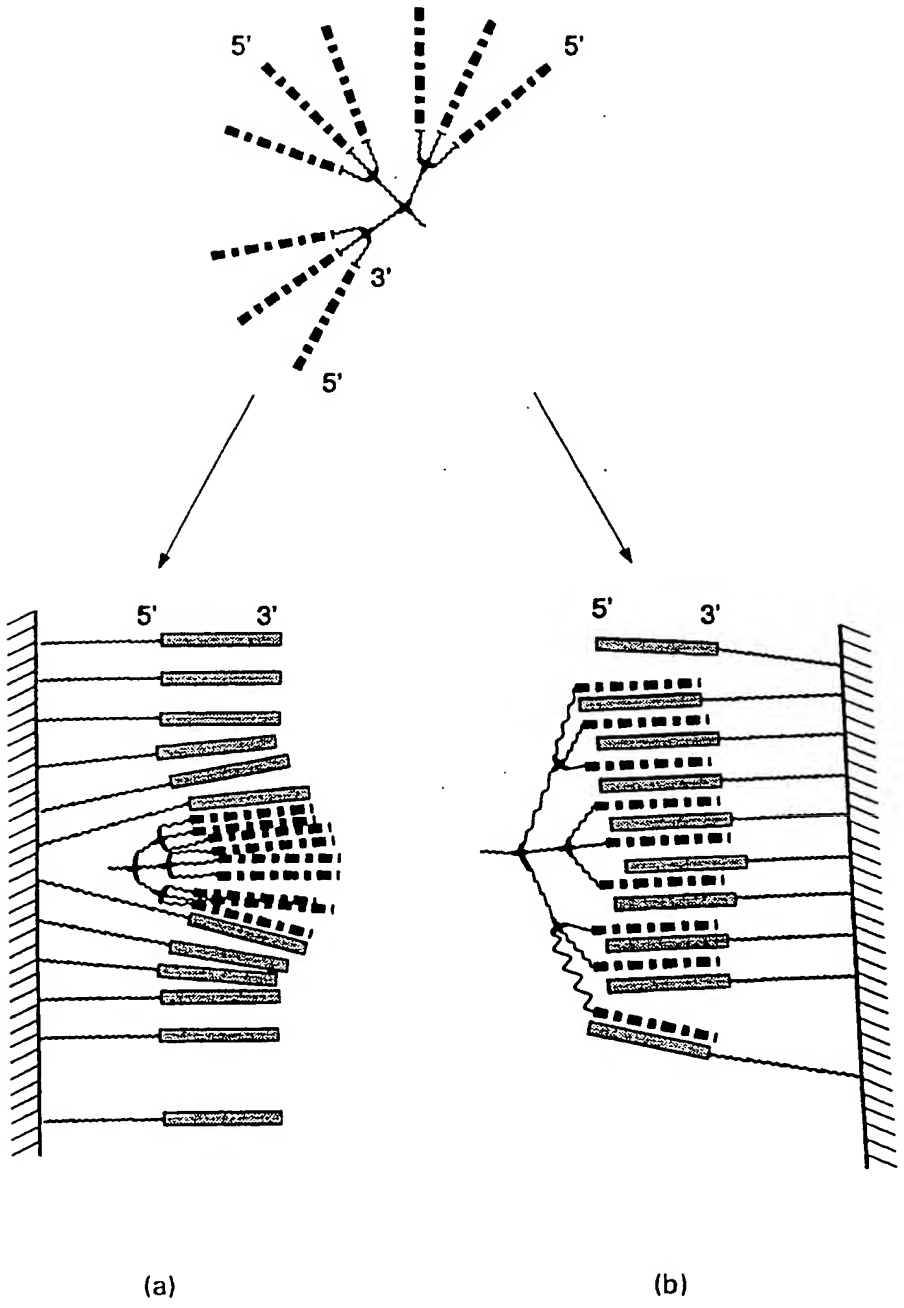

% of target dissociated
100
50 (Tm)
association
dissociation
Temperature
single-stranded polynucleotide
dendrimer

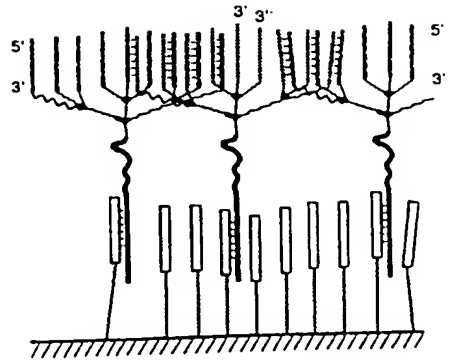
Fig. 1(b)

Fig. 2

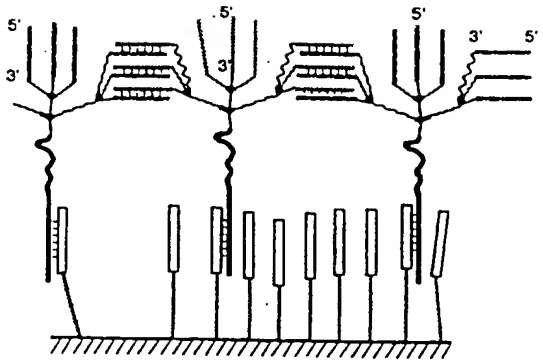


(a) (b)

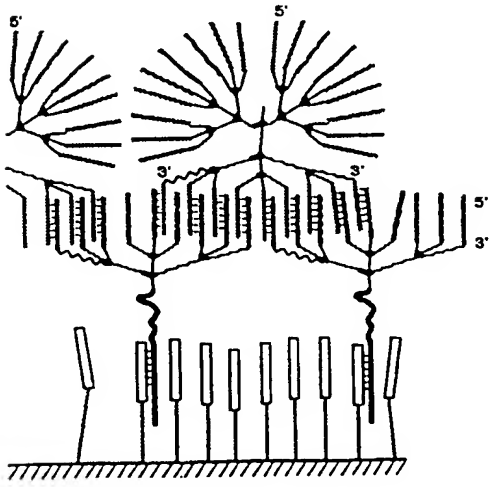
Fig. 3



(a)



(b)



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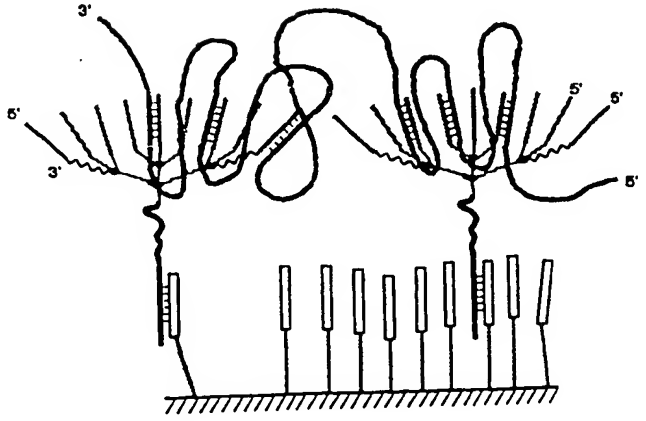

(d)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Isis Innovation Limited
(B) STREET: University Offices, Wellington Square
(C) CITY: Oxford
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): OX1 2JD

(ii) TITLE OF INVENTION: POLYNUCLEOTIDE MULTIMERS AND THEIR USE IN HYBRIDISATION ASSAYS

(iii) NUMBER OF SEQUENCES: 5

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(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: GB 9811403.6

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTCTTTCTCT TCCCT 15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTTCTCTTTC TCTTC 15

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCTCTTTCCC TTCCTTCCTC 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TACAGCAAAT GCTTGCTAGA CCAATAATTA GTTATTCACC TTGCTAAAGA AATTCTTGCT 60

CGTTGACCTC CACTCAGTGT GATTCCACCT TCTCCAAGAA CTATATTGTC TTTCTCTGCA 120

AACTTGGAGA TGTCCTA 137

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGTGGAGGTC AACGAGCAAG AATTTCTTTA GCAAGG 36

INTERNATIONAL SEARCH REPORT

	ational Application No
	PCT/GB 99/01697

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 175 270 A (T.W.NIELSEN ET AL.) 29 December 1992 (1992-12-29) column 2, line 60 -column 4, line 14 column 12, line 49 -column 14, line 33 column 15, line 67 -column 16, line 47; claims ---	1-14
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 October 1999	03/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luzzatto, E

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	M.S.SHCHEPINOV: "Oligonucleotide dendrimers: synhesis and use as polylabelled DNA probes" NUCLEIC ACIDS RESEARCH, vol. 25, no. 22, 1997, pages 4447-4454, XP002082740 uk the whole document ---	1,2,4,6, 8,9
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